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Searching for Novel Fungal Biological Control Agents for Plant Disease Control Among Endophytes

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Abstract

There are increasing efforts aiming to utilise endophytes as biological control agents (BCAs) to improve crop production. However, reliability remains a major practical constraint for the development of novel BCAs. Many organisms are adapted to their specific habitat; it is optimistic to expect that a new organism added can find a niche or even out-compete those adapted and already present. Our approach for isolating novel BCAs for specific plant diseases is therefore to look in healthy plants in a habitat where disease is a problem, since we predict that it is more likely to find competitive strains among those present and adapted. *In vitro* inhibitory activities often do not correlate with *in planta* efficacy, especially since endophytes rely on intimate plant contact. They can, however, be useful to indicate modes of action. We therefore screen for *in planta* biological activity as early as possible in the process in order to minimise the risk of discarding valuable strains. Finally, some fungi are endophytic in one situation and pathogenic in another (the mutualism–parasitism continuum). This depends on their biology, environmental conditions, the formulation of inoculum, the health, developmental stage and cultivar of the host plant, and the structure of the plant microbiome.

2.1 Introduction

It is a major challenge to increase crop yields worldwide in a sustainable manner. There is an increasing wish to reduce the use of pesticides, the application of inorganic fertiliser and the need for irrigation, as these factors deplete natural resources and can have negative effects on the environment. We need to meet the challenges of climate change, as well as world population growth and other demographic factors, such as urbanisation and economic growth. The combination of these factors results in increased demand for both water resources and meat, hence of plants as food and fodder. Together, these many factors place high demands on agriculture, motivating research effort to improve yields in a sustainable manner: the global challenge is to produce more food from less agricultural land with reduced inputs.

Plant diseases reduce yield and profitability, both in terms of direct losses, product quality and indirectly through inputs into the crop. However, the grower has many practical tools for combating plant diseases, including cultural practices such as crop rotation, fertilisation, pruning, irrigation and tillage. Chemical control agents are also of seminal importance for many diseases: these can be directly antimicrobial, e.g. fungicides, or act as inducers of plant defence responses and therefore disease resistance, e.g. benzothiadiazole (BTH, Bion®) and probenazole. However, there are increasing problems with fungicide resistance in many important pathogens, which means that some available fungicides have become ineffective for specific diseases (Lucas *et al.*, 2015). Furthermore, the number of approved fungicides is being reduced due to side effects, e.g. accumulation in the environment, conflicts with medicinal use (Berger *et al.*, 2017); at the same time few new products are being developed and approved.

Another important strategy is the use of disease resistance. This can be achieved through conventional plant breeding by introducing resistance genes from within the same plant species (e.g. from landraces) or closely related plant species. Effective sources of disease resistance are often unavailable for conventional plant breeding programmes, especially for necrotrophic pathogens. Resistance can also be achieved through biotechnological approaches, e.g. genetic engineering, to add genes which encode either antimicrobial agents or regulators of defence mechanisms (Collinge *et al.*, 2010, 2016). More recently, an increased understanding of the biology of microbial pathogenicity has led to new breeding technologies, which can manipulate pathogens, for example, by host-induced gene silencing (HIGS) or by using gene editing techniques (site-directed mutation), e.g. CRISPR-Cas9 (Nowara *et al.*, 2010; Collinge, 2018). Even the combination of these approaches is insufficient to control many important diseases of key crops and, furthermore, there is a reluctance to use the new breeding technologies in some parts of the world (Collinge, 2018). There is a need to be innovative in finding new approaches, especially since many important pathogens are well able to adapt to, and can therefore overcome, both

disease resistance and pesticides (e.g. *Zymoseptoria tritici*, *Phytophthora infestans*; Lucas *et al.*, 2015).

An underdeveloped and underutilised means of disease control is biological control. Biological control is defined as direct or indirect inhibition of a disease or the pathogen causing the disease, by another organism (antagonist) or group of organisms. Biological control is seen as an increasingly attractive tool to complement the more traditional control strategies both in organic and conventional agriculture. Furthermore, biological control can be especially useful when used in organic agriculture, as well as in systems where disease resistance is unavailable in the host and where fungicide resistance is prevalent in the pathogen populations. Some biological control organisms (BCAs) can even supplement fertilisers to stimulate plant growth (Coleman-Derr and Tringe, 2014; Jensen *et al.*, 2016a). The exploitation of beneficial microorganisms and the management of the plant microbiome can contribute to these needs, both through biological control such as by stimulation of host growth. Although development of a product may be achieved without understanding how it works, there are many fascinating fundamental questions about the nature and physiological mechanisms underlying plant–microbe interactions which beg study (Latz *et al.*, 2018). There are also potential risks associated with biological control such as the negative consequences of introducing organisms into new habitats and production systems which need to be considered before permitting the use of a new biological control agent. For these reasons, the regulation of commercialisation of novel BCAs is stringent, and therefore expensive (Anon, 2009).

As a division of the plant microbiome, endophytes represent a largely unexploited source of organisms with potential for the biological control of pests and pathogens and for helping plants adapt to abiotic stress. One of the main drawbacks of biological control is inconsistency under variable environments. Since the endophyte is inside the plant, it is potentially better protected from the external environment and, therefore, we consider it likely that at least some endophytes will prove to be more reliable BCAs than epiphytic phyllosphere or rhizosphere organisms. Here, we provide a resumé of current knowledge of the similarities and differences between commensal, mutualistic and pathogenic interactions between plants and microorganisms, with emphasis on different approaches for isolating and studying endophytic fungi. We favour an ecological approach, namely isolating BCAs from plants which are coping with a stressful environment. We isolate from different plant organs (and mutants), to which BCAs can be returned and utilised for plant disease control. Another approach being taken, which we do not address here, is to try to manipulate microbiomes rather than using individual organisms, e.g. by manipulating agricultural practice and choice of cultivar. This is the particular focus of studies using bacterial microbiomes (Finkel *et al.*, 2017), but we predict an increase in studies on fungal microbiomes will follow.

2.2 What Is an Endophyte?

Endophytes (*endo* = within, *phyto* = plant), a term coined by the father of plant pathology Anton de Bary in 1886, are now defined as microorganisms capable of colonising inner parts of plants without causing disease symptoms (Schulz and Boyle, 2005; Kusari *et al.*, 2012; Wani *et al.*, 2015). The plant is not host to a single microorganism, but hosts many different microorganisms simultaneously; collectively, these are termed the endomicrobiome. The endomicrobiome is diverse and comprises, e.g. archaea (Moissl-Eichinger *et al.*, 2018), bacteria (Furnkranz *et al.*, 2012; Aguiar-Pulido *et al.*, 2016) and diverse fungi including both ascomycetes (Newsham, 2011) and basidiomycetes (Weiß *et al.*, 2016; Hardoim *et al.*, 2015), as well as some endophytic oomycetes (Ploch and Thines, 2011). We have much to learn about the individual microorganisms and their interactions in ecological communities of microorganisms comprising the endomicrobiome.

A specific organism might be endophytic in one situation and saprotrophic, epiphytic or even pathogenic in others (Hardoim *et al.*, 2015; Mukherjee *et al.*, 2012). In the latter case, there is the issue of whether it is possible to distinguish whether an organism is endophytic or a quiescent or latent pathogen (Louarn *et al.*, 2013; Zeilinger *et al.*, 2016; Lofgren *et al.*, 2018). Indeed, the term endophyte covers different ecological strategies (latent pathogen, beneficial symbiont, commensal passenger) used by different organisms in different situations (species or growth conditions), which result in what we observe as the endophytic habit (Schulz and Boyle, 2005). It can be difficult to assign a particular interaction one of these specific biological terms and this situation is encompassed by the concept of 'mutualism–parasitism continuum' (Müller and Krauss, 2005; Schulz and Boyle, 2005).

Different strains of specific taxa (e.g. *Fusarium oxysporum*) can be endophytic BCAs or pathogens on the same host. Quite closely related taxa can include both pathogens or endophytes (Fravel *et al.*, 2003; Ma *et al.*, 2010). Some organisms are pathogens of one host, and endophytes in another closely related host species, and can thus exhibit different roles, ranging from latent pathogens or endophyte to pathogen, e.g. *Colletotrichum* spp. and *Fusarium* spp. (De Silva *et al.*, 2017; Lofgren *et al.*, 2018). In contrast, *Ramularia collo-cygni* is an endophyte in wheat but a pathogen in barley under certain physiological conditions or genetic backgrounds (McGrann *et al.*, 2014, 2016). *Fusarium graminearum* is a pathogen of small grain cereals, but apparently an endophyte in other species of grass (Lofgren *et al.*, 2018). What we do not know is whether they are commensals, deriving nutrition or other benefits without causing measurable harm (i.e. quiescent pathogens), and therefore technically not symbionts according to our narrow definition, or whether they are, in fact, beneficial symbionts in these alternative hosts. Furthermore, some pathogens arguably exhibit an endophytic growth habit in the first part of the infection cycle and cause disease later, e.g. *Zymoseptoria tritici* (formerly *Septoria tritici*

(anamorph) *Mycosphaerella graminicola* (telomorph)) (Shetty *et al.*, 2003; Sánchez-Vallet *et al.*, 2015). Indeed, it is perhaps useful to exclude the potential pathogens from the definition and confine the term to organisms that, in a specific situation, have a mutualistic or at least commensal relationship with their host. An adapted version of Koch's postulates can be useful to exclude organisms which cause disease when reintroduced into the host (Card *et al.*, 2016).

Genotype can also be important, thus many strains of *Fusarium oxysporum* are endophytic, but possession of some 'B chromosomes' can confer pathogenicity (Ma *et al.*, 2010). Does the plant decide whether the organism is a pathogen or endophyte by the way it reacts to the colonising organism or is the interplay more complex? Interestingly, there is increasing evidence to suggest that some effectors play a role in competitive fungal–fungal interactions (Rovenich *et al.*, 2014). Furthermore, some endophytes can not only induce resistance but, at least in some cases, susceptibility to pathogens (Houterman *et al.*, 2008; Kurose *et al.*, 2012). Thus, the same endophyte may influence different interactions in opposite ways.

2.3 How Do We Find Endophytes?

There are two major approaches to identifying endophytes in a plant: cultivation-dependent (e.g. isolation) and cultivation-independent (e.g. metagenomics) methods. Each approach has advantages and disadvantages (Figure 2.1). It is important that the tissue from which endophytes are isolated is fresh and shows few or no visible disease symptoms. To ensure that organisms isolated are endophytic and not epiphytic, the tissue needs to be surface sterilised. This is especially important for roots and tubers. This can be achieved in several ways (e.g. alcohol, sodium hypochlorite treatment) and there is a compromise between using too harsh a treatment, which penetrates the tissue killing the microorganisms within, and too gentle a treatment, which allows too many epiphytic or rhizospheric organisms to survive (Busby *et al.*, 2016b). Standardising these conditions before working with a new species or tissue is highly recommended as harsh treatments can reduce fungal survival inside the tissue or mild treatments can result in false positives.

Clearly, if an organism is to be used in crop production, it has to be cultivable. Many endophytes are not. In order to increase the chances of isolating, hence cultivating and utilising endophytic microorganisms, the growth media can be supplemented with plant extracts. This has been shown to increase the number of endophytes recovered (Eevers *et al.*, 2015; Murphy *et al.*, 2015). In our experience, plant-extract-complemented media do not improve yield or diversity in the isolation of endophytes from cereals (Latz *et al.*, unpublished). However, different fungi have more or less specific requirements for the media that they will grow on, and, furthermore, the isolation process itself may inhibit the growth of some fungi, since

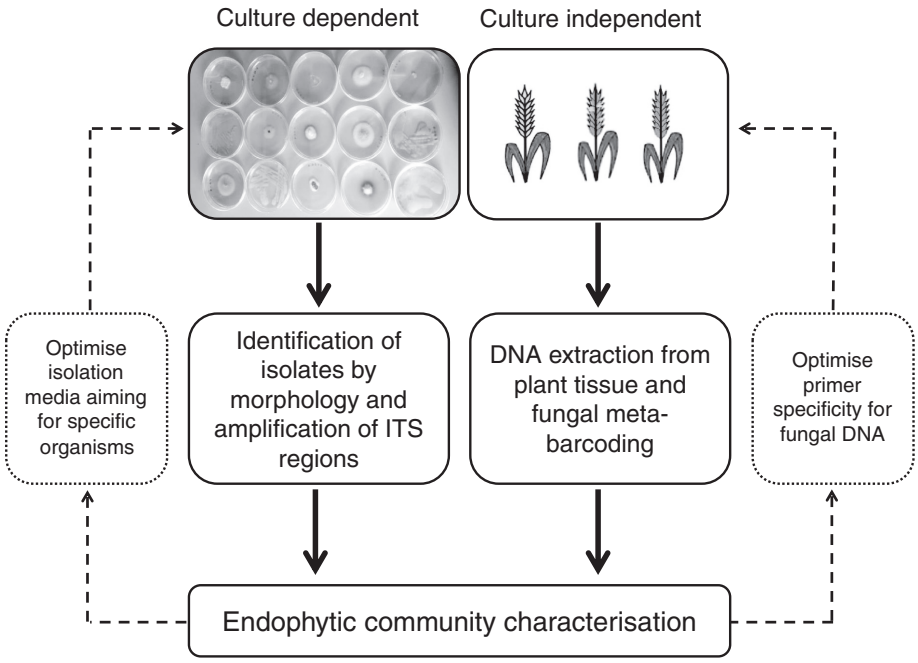

Figure 2.1 Endophytic community characterisation using culture-dependent and culture-independent methods. Both methods can be optimised in order to obtain a more accurate description of fungal microbiome.

antimicrobial compounds can be induced and/or released from the host tissues on damage. Moreover, different microorganisms grow at different rates and some will simply be outcompeted by others. As this is especially a problem with bacteria outcompeting fungi, antibacterial compounds may be incorporated in media when the target endophyte is fungal, although this may not itself be without negative effect. Low nutrient media can favour slow-growing fungi in relation to fast-growing fungi and bacteria. In conclusion, isolation-based approaches have little value in quantitative floristic diversity assessments, but they remain our only door to finding potential biocontrol agents. Additionally, it is important to standardise different techniques and growth media for isolation in each case. Once isolated, the fungi can be identified by morphological traits combined with marker gene sequencing (typically the ribosomal genes).

Metagenomics as a methodology can be used to obtain a more objective and quantitative, although still descriptive, assessment of the endophytic fungal microbiome *in situ* (Diaz *et al.*, 2012; Tian *et al.*, 2015; Abdelfattah *et al.*, 2016; Sapkota *et al.*, 2017). The information obtained may even be used as a guide for choosing specific media for targeted isolation of specific taxa that appear to be enriched in the microbial community. For fungal endophytes (and indeed most

eukaryotes), the most common way to perform a fungal community analysis is to amplify parts of the ribosomal genes, for example, the internal transcribed spacer (ITS)-1 (Nicolaisen *et al.*, 2014) or ITS-2 (Hertz *et al.*, 2016; Gdanetz and Trail, 2017), followed by deep-sequencing and bioinformatical analyses in order to investigate the composition of the endophytic communities without the need to isolate living fungi from the host plant. The main challenge with this approach is finding primers which recover most of the fungal diversity without creating phylum biases, while at the same time avoiding targeting the host genome. This has been a constant problem, especially in polyploid species such as wheat. Many pairs of primers have been suggested using *in silico* analysis, but no real comparison has been performed on DNA samples (Toju *et al.*, 2012). Compromises will therefore be necessary!

Shot-gun metagenomics or whole metagenome shotgun sequencing concerns deep sequencing of the mixed genomes of the organisms present in the sampled material (Aguiar-Pulido *et al.*, 2016; Kaul *et al.*, 2016). This approach, initially applied for the study of bacterial endomicrobiomes (Bulgarelli *et al.*, 2012; Lundberg *et al.*, 2012; Hardoim *et al.*, 2015), eliminates the bias unavoidably introduced when selecting amplification primers since the sequencing process itself is objective. The technique is, however, currently more expensive, both in terms of the chemical process and in the need for computing time during bioinformatics analysis; huge amounts of sequence data are generated, which have to be analysed. Thus, the quality of the genome sequence databases is a constraint on the quality of the analysis: although the number of sequenced genomes in the databases increases with time, the extent and quality of annotation of the sequences in them lags, and a large proportion of the data therein are not yet assigned to specific organisms.

A third approach, metatranscriptomics, uses RNA as the substrate for sequencing, i.e. gene transcripts rather than the genome (Aguiar-Pulido *et al.*, 2016; Kaul *et al.*, 2016). This means that the results reflect the physiological activity of organisms and therefore give more information about the actual plant microbiome interactions, and not just an image of the microbial communities within the plant (Kaul *et al.*, 2016). This approach will also indicate which gene families participate in or are necessary for the endophytic lifestyle, for example, genes involved in hormone production or action, effector proteins and specialised metabolites (see below). The value of the analysis depends on the choice of physiological conditions; an important component of the microbiota may have latent infection periods and may therefore be unrepresented in the results.

It should, at least to some extent, be possible to combine these approaches in order to use amplicon sequencing and whole metagenome sequencing (and metatranscriptomics) to identify endophytes with desired traits (functions or e.g. metabolites), and chose, or even design, isolation methods tailored to their real or perceived needs, if such interesting organisms are indeed culturable. Depending

on the stamina of the researcher, the classical microbiological approach can easily yield 40–120 fungal taxa per sample, e.g. (Comby *et al.*, 2016; Kernaghan *et al.*, 2017; Kosawang *et al.*, 2018), and amplicon sequencing approaches typically reveals 200 or more within a study (Nicolaisen *et al.*, 2014).

2.4 What Do Endophytes Do to the Plant?

Endophytes can support growth and health of the host plant via different physiological mechanisms. Thus, some fungal endophytes can stimulate abiotic stress tolerance (e.g. drought and salt stress) and plant growth by altering hormone balance (see below) or nutrient acquisition (Zeilinger *et al.*, 2016). Superficially, it may seem surprising that some endophytes do this, but there is no need to look for a more complicated reason for why this is, than that a happier plant can support a higher biomass of a specific endophyte. In other words, in an ecological sense, the endophyte may find a safe niche within the host tissue (protection and reduced competition for nutrients), while improving the plant's performance under specific stress conditions. Survival and proliferation of the endophyte may simply depend on growth and survival of the host. These are by no means universal properties of endophytes, and the majority may only be neutral commensal passengers which do not measurably influence their host. Furthermore, the ability to stimulate plant growth is by no means exclusive to endophytes as plant growth-promoting organisms are also found among rhizosphere microorganisms and mycorrhizal fungi.

2.5 How Do Pathogens and Endophytes Differ?

Evidence coming from overlapping transcription profiles of endophyte- and pathogen-colonised plants indicates a level of similarity between the host response to colonisation and infection, at least at the initial stages of both types of interactions (Guimil *et al.*, 2005; Schäfer *et al.*, 2009; Zamioudis and Pieterse, 2011). In other words, at least some endophytes can induce plant immune responses through microbe-associated molecular patterns (MAMPs) that are recognised by the plant in a similar way to those produced by pathogens (Latz *et al.*, 2018). It has long been speculated that, as for pathogens (Lo Presti *et al.*, 2015), one strategy used by endophytes to avoid detection is to supress plant immunity using effectors (Sánchez-Vallet *et al.*, 2013; Rovenich *et al.*, 2014), and this can even result in induced susceptibility to pathogens (e.g. Kurose *et al.*, 2012; Busby *et al.*, 2016a). So, what are the actual differences between endophytes and pathogens? Is the intention of the pathogen necessarily bad, or is it just a question of compatibility, i.e. expression of genes often associated with pathogenicity such as those encoding cell

wall degrading enzymes? The fact that at least some organisms can behave as an endophyte in one situation and as pathogen in others suggests that there is: (1) a continuum from beneficial to harmful (and vice versa); and (2) no simple answer to the question 'How do pathogens and endophytes differ?'

The complexity of the mechanisms of recognition and response to fungi can be illustrated with a pathogen-related example, which may be predicted to apply to endophytes too: chitin is a MAMP where analyses of plant–microorganism interactions have demonstrated several layers of interaction (signal and reaction, counter-reaction, new signal and so on). *Passalora fulva* (formely *Cladosporium fulvum*) is considered to be a pathogen of tomato which displays an initially endophytic lifestyle (traditionally considered to be biotrophic by some authors) in the apoplast without making specific infection structures. Several seminal studies have demonstrated multiple layers of protection used by this pathogen to avoid being recognised by the host or to counter the triggered immune response: (1) Fungal effectors (chitin-binding lectins, including Avr4) coat the hyphal surface and act as inhibitors of the plant defence enzyme chitinase (van den Burg *et al.*, 2006). (2) The effector Avr4 is recognised by the host receptor Cf4 (a resistance gene) triggering immunity (i.e. ETI). The CERK receptor-like protein kinase (3) binds chitin fragments released by host chitinase to enhance the induced immunity, but (4) the pathogen produces the effector Ecp6 to scavenge chitin fragments (de Jonge *et al.*, 2010; Malinovsky *et al.*, 2014).

2.6 Hormones in Plant–Endophyte Interactions

It is well established that hormones, such as abscisic acid (ABA), auxins (e.g. indole acetic acid, IAA), brassinosteroids, cytokinins (many), ethylene, gibberellins, jasmonates (JAs), salicylic acid (SA) and strigolactones are important for plant development and modulation of plant defences against pathogens (Pieterse *et al.*, 2012; De Vleesschauwer *et al.*, 2013; Großkinsky *et al.*, 2016; Ma and Ma, 2016). Pathogens (and presumably also endophytes) modulate hormone levels using several mechanisms. Thus, they can (1) stimulate the host to increase or decrease the production of specific hormones; (2) produce and degrade hormones themselves; (3) insert biosynthetic genes for phytohormones into the host chromosomes; and (4) produce molecules which emulate hormone action. Examples are emerging that many hormones, e.g. auxins (Hilbert *et al.*, 2012), JAs, gibberellins (Schäfer *et al.*, 2009; Jacobs *et al.*, 2011), ABA (Peskan-Berghöfer *et al.*, 2015), ethylene (Khatabi *et al.*, 2012) and SA (Alonso-Ramírez *et al.*, 2014) play a role in regulating the ability of endophytes to colonise the host tissue.

Several hormones are involved in regulating the establishment and maintenance of infections with the model root endophyte *Serendipita indica* (formerly

Piriformospora indica) which can form associations with many important crop plants, e.g. wheat, barley and tomato (Weiß *et al.*, 2016), and model plants, i.e. *Arabidopsis* (Stein *et al.*, 2008) and *Brachypodium* (Ye *et al.*, 2014). *Serendipita indica* is considered to be a root endophyte, although it does induce necrosis at the site of colonisation. *Serendipita indica* has been developed as a model endophyte through the combination of wide host range and its positive effects on many hosts. Experimentally, a fairly well annotated genomic sequence and molecular genetic tools are available, since the fungus can be cultured on artificial media (Zuccaro *et al.*, 2009, 2011). Studies in *Arabidopsis* using the JA-insensitive host mutants *jai1* and *jar1* demonstrated that *S. indica* lost the ability to supress host immunity. On the other hand, mutants involved in gibberellin signalling, a *della* mutant in which gibberellin levels are high and the *ga1–6* mutant (impaired in biosynthesis), exhibited higher and lower levels of infection, respectively, compared to the wild-type plants (Jacobs *et al.*, 2011). The effects of auxin on *S. indica* infection were studied in barley (Hilbert *et al.*, 2012) using gene silencing to compromise auxin production in the fungus: these strains showed reduced rates of early infection of barley roots, but overall levels of infection were unaffected. JA signalling is often related to ethylene signalling and it has been shown that ethylene production is induced by *S. indica* in *Arabidopsis* and barley (Khatabi *et al.*, 2012). *Arabidopsis* mutants with increased ethylene production and ethylene-induced defence mechanisms were more susceptible to *S. indica* infection. *S. indica* treatment of barley resulted in the induction of the expression of genes involved in the metabolism of several hormones including the biosynthesis of auxins, brassinosteroids and gibberellin as well as ABA responsive genes (Schäfer *et al.*, 2009). The possible contribution of hormones produced by the fungus was not taken into account in this study, as described above. However, other studies have demonstrated hormone production by fungi (Hilbert *et al.*, 2012; Khan *et al.*, 2012; Waqas *et al.*, 2015).

The effects of altered hormone levels are both subtle and complex; indeed, the same hormones can stimulate defence or pathogenicity in interactions with different pathogens, and different hormones can modulate each other's effects by complex crosstalk (Pieterse *et al.*, 2012). Most studies address individual hormones in isolation. In some cases, the concerted action (crosstalk) between hormones, e.g. IAA and gibberellins (Waqas *et al.*, 2012), has been demonstrated. Indeed, some endophytes and other beneficial fungi (and bacteria) produce or induce the production of specific plant hormones to modulate this crosstalk for their benefit and often also for the host plants' benefit (Schäfer *et al.*, 2009; Evangelisti *et al.*, 2014; Gutjahr, 2014). Techniques have been developed that allow monitoring of several hormones simultaneously (Kojima *et al.*, 2009; Ionescu *et al.*, 2017). Only by doing this, ultimately, it will be possible to unravel the roles, effects and the mode of action of hormones exploited in the interactions between endophytes and plants under different physiological conditions. We still have much to learn!

2.7 Effectors in Plant–Endophyte Interactions

Effectors are traditionally defined as pathogen proteins introduced into the host cell in order to repress host defence (plant immunity). Indeed many, especially hemibiotrophic/biotrophic filamentous pathogens such as the oomycete *Phytophthora infestans* and ascomycete *Blumeria graminis*, have a huge arsenal of effector proteins for this purpose (Ahmed *et al.*, 2016). Plants need to defend themselves from attack and, taxonomically, endophytes are often related to pathogens, producing the same MAMPs (that can trigger defence, MTI or MAMP-triggered immunity) (Collinge *et al.*, 2016). It is necessary for endophytes to avoid triggering the induction of plant defences, and effector proteins are among the tools needed to do this, indeed candidate effectors are now being found in some endophytes. For instance, *S. indica* uses a battery of effectors to suppress host defences (Jacobs *et al.*, 2011; Rafiqi *et al.*, 2013), although the expression of many defence-related genes is induced (Schäfer *et al.*, 2009). Studies comparing plant responses to pathogenic and endophytic organisms do indicate that both are recognised in the same manner. However, they may differ in induction of the full defence response, because the endophytic organisms lack some unidentified pathogenic determinants (Wani *et al.*, 2015; Xu *et al.*, 2015). Thus, although the process of initial recognition is the same for both endophyte and pathogen, downstream regulatory mechanisms must be different. The question is therefore, what are these mechanisms? Are they determined by the endophyte or the plant, or more likely by crosstalk between the two (Kusari *et al.*, 2012)?

2.8 Specialised or Secondary Metabolites in Plant–Endophyte Interactions

It is well known that plants produce compounds, termed specialised (traditionally termed secondary) metabolites, collectively known as phytoalexins or phytoanticipins, to prevent or restrict the growth of fungi within the tissue (Ludwig-Müller, 2015; Rook, 2016). On the other hand, many pathogens produce phytotoxins to facilitate infection, i.e. metabolites toxic to plants. Many endophytes are also capable of inducing plant-based production of specialised metabolites upon colonisation. These metabolites have roles in competition between microbes and in some cases communication (Kusari *et al.*, 2012; Dupont *et al.*, 2015). The diversity of specialised metabolites produced in plant–microbe interactions is enormous and the range includes alkaloids, polyketides, terpenoids, phenylpropanoids, flavonoids, steroids, quinones, xanthones, benzopyranones, tetralones, cytochalasines and enniatines. Despite the inherent limitations in trying to study chemically diverse compounds simultaneously, the technologies of metabolomics for the study of the

entire cellular diversity of these metabolites has improved substantially in recent years (Tenenboim and Brotman, 2016). A major role for these metabolites is to help one or the other partner in competitive interactions with other organisms they encounter, these can be: (1) other microorganisms they compete with; or (2) their hosts. Specific compounds play an important role in plant–endophyte communication and therefore in the establishment of a successful interaction (Kusari *et al.*, 2012). Specialised metabolic pathways of host plant and endophyte can interact in many ways (Kusari *et al.*, 2012). The metabolism of the host plant and/or endophyte can be influenced by the other partner (Ludwig-Müller, 2015): (1) the endophyte may induce host biosynthetic activity; (2) the host can induce endophyte biosynthetic activity; (3) the host and endophyte may share parts of a specific biosynthetic pathway and thus each may contribute partially to producing novel specialised metabolites; and (4) both the host and/or the endophyte may metabolise products made by the other partner.

Compared to other types of microbial interactions, our knowledge of the metabolites from endophyte interactions is both rudimentary and of great interest as a source of new metabolites with industrial potential, not least for the pharmaceutical industry (Kusari *et al.*, 2012). Many studies have demonstrated that pathogens adapted to a particular host are well capable of dealing with the phytoalexins and phytoanticipins produced in their host (Rook, 2016). This is apparently also true for endophytic fungi. For example, the majority of isolated oat root endophytes were resistant to the phytoanticipin avenicin, a saponin (i.e. terpenoid derivative) (Carter *et al.*, 1999). This raises an issue: one of the strategies for achieving transgenic disease resistance against pathogens is to move the biosynthetic apparatus for specific phytoalexins and phytoanticipins to plant species where the pathogens have not evolved to overcome them. This may have undesirable negative effects on the endophytic microbiome of the recipient since the natural endophytes adapted to a particular species would not be adapted to the phytoalexins and phytoanticipins originating from the gene donor, and this potential negative effect should be looked for when using this plant protection strategy.

It is also well known that many fungi produce specialised metabolites to give them a competitive advantage in interactions with other fungi or their hosts. Where these metabolites are measurably toxic for the host, they are termed phytotoxins. By definition, an organism behaving as an endophyte does not produce phytotoxins while exhibiting this lifestyle since this defines the necrotrophic phase of a fungal infection (Howlett, 2006). Endophytes may nevertheless produce secondary metabolites for their own benefit, e.g. to suppress the plant's defence system (which represents a conceptual overlap with hormones) or growth of competitors within the plant. In the case of the ryegrass endophyte *Epichlöe festucae*, a massive host response was induced at the level of induced gene expression, and much could be attributed to the production of specialised metabolites at the expense of primary metabolism

(Dupont *et al.*, 2015). The presence of several different endophyte species can lead to metabolite production by endophytes and/or host, because of competition (Kusari *et al.*, 2012). For example, the specialised metabolite production (specifically polyketide biosynthesis), differed in the endophyte *Alternaria tenuissima* depending on the presence of another endophytic fungus, *Nigrospora sphaerica*, in an *in vitro* system where the former inhibited growth of the latter (Chagas *et al.*, 2013). Sometimes, the metabolites shift the balance in the mutualistic interaction, turning the endophyte into a pathogen (Schulz and Boyle, 2005). Specialised metabolites produced by endophytes can be used by the host. For example, *Epichlöe* species produce ergot alkaloids which protect the plant against herbivores (Schardl and Phillips, 1997).

2.9 Selecting Endophytes as Potential BCAs

Many researchers use high-throughput *in vitro* methods to screen for potential biocontrol agents by isolating cultivatable organisms and test for their potential as BCAs. Typically, *in vitro* confrontation assays (dual culture) are performed to look for the ability of the potential BCA to inhibit growth of the target pathogen directly, i.e. direct antimicrobial activity (Zachow *et al.*, 2008; Furnkranz *et al.*, 2012; Gdanetz and Trail, 2017; Kosawang *et al.*, 2018) before moving on to *in planta* assays. The *in vitro* approach is often successful as evidenced by numerous reports of seemingly successful identification of new BCAs where this approach was employed. However, drawbacks of using this *in vitro* approach are: (1) they often do not correlate with disease control efficacy *in planta*; (2) the mechanism of induced resistance cannot be discovered in the absence of the plant. So, even if the *in vitro* approach is seemingly successful, it is not known how many potentially effective strains were discarded in the screening process. Despite these drawbacks, *in vitro* testing is often used for high-throughput screening of existing collections. In contrast to the *in vitro* approach, and in common with others (Schisler and Slininger, 1997; Comby *et al.*, 2016; Zhao *et al.*, 2017; Kernaghan *et al.*, 2017), our approach is ecological or niche-based: to look for novel fungal BCAs in the habitat where they can interact with pathogens (Figure 2.2). Specifically, the idea is to look for more healthy plants (i.e. less stressed) in these habitats to test the hypothesis that these plants manage better due to the presence of a biocontrol microorganism in their microbiome. In other words, our thesis is that endophytes isolated from the environment where they are intended for use are more likely to be adapted to these specific environmental conditions (e.g. water availability, temperature, UV radiation, competing organisms, etc.).

There are relatively few examples of beneficial endophytic fungi identified using niche-based screening strategies (Lugtenberg *et al.*, 2016; Kernaghan *et al.*, 2017; Zhao *et al.*, 2017). On the other hand, there are several examples of effective fungal

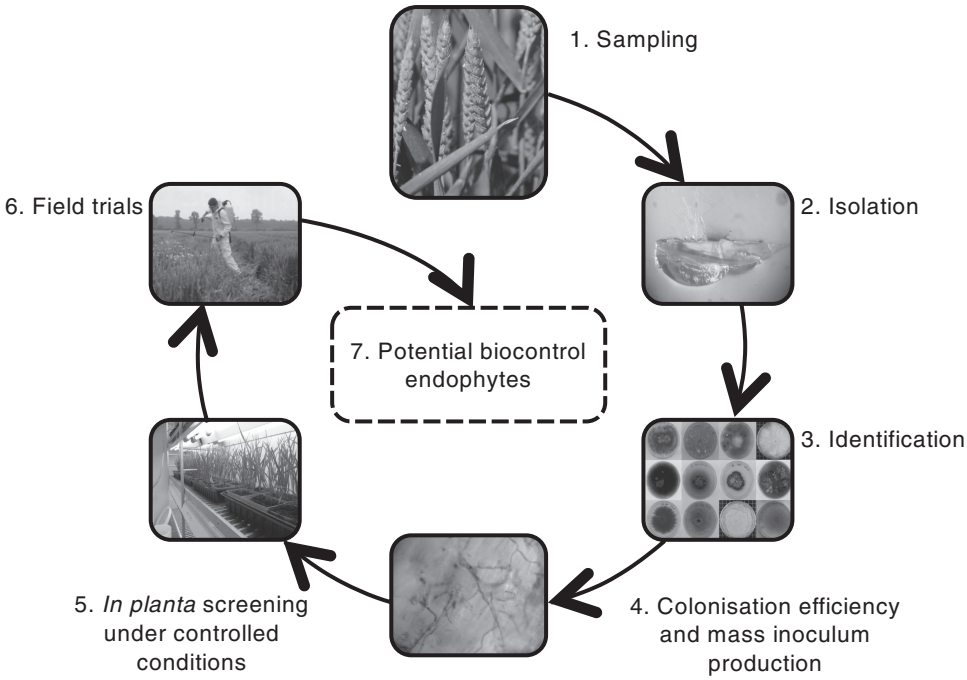

Figure 2.2 The process of discovery of biocontrol endophytes: 1. Sampling healthy plants in areas under disease pressure. 2. Isolation on artificial media from surfaced sterilised tissues (wheat glume). 3. Molecular identification of isolates using ribosomal genes. 4. Colonisation efficiency and mass production tests can reduce the number of potential isolates, as well as detect latent pathogens. 5. Screening of endophytes against diseases in small *in planta* assays provides a better observation of potential candidates. 6. Successful isolates can be tested in field conditions on larger-scale experimental units in order to assess field consistency. 7. Potential biocontrol endophytes can enter an industrial phase or mass production and marketing or continued biological studies about their mode of action and ecological significance. (A black and white version of this figure will appear in some formats. For the colour version, please refer to the plate section.)

BCAs from other microbiome components. An antagonistic isolate of *Cladosporium cladosporioides* was isolated from a sporulating colony of *Venturia inaequalis* and was shown to be as effective as fungicides for the control of apple scab in the field (Köhl *et al.*, 2014). The fungus *Clonostachys rosea* was originally isolated from *Fusarium*-infected barley roots (Knudsen *et al.*, 1995) and control of seed-borne and root-infecting diseases was demonstrated in the field (Jensen *et al.*, 2000). However, the exceptions prove the rule: *C. rosea* is also effective in controlling diseases in carrot (Jensen *et al.*, 2004; Koch *et al.*, 2010), Chinese cabbage (Møller *et al.*, 2003), oak (Knudsen *et al.*, 2004) and strawberry (Mamarabadi *et al.*, 2008), as well as foliar diseases in cereals (Jensen *et al.*, 2016a), demonstrating that it is

competitive in habitats other than those it was isolated from. Another example is fungal root endophyte *S. indica*, which also exhibits a broad host range and acts as an effective BCA against several diseases (Franken, 2012; Card *et al.*, 2016).

Having isolated the organisms, a common approach is to design assays for biological control, which resemble the conditions where the potential BCAs will be used. This is done by screening the organisms *in planta* against pathogens under controlled conditions with an adequate number of replications since BCAs tend to show variable efficiency. However, for some pathosystems, large-scale *in planta* screening may not be feasible, for example due to the limited availability of material or timescale for bioassays (Kosawang *et al.*, 2018). Small-scale field experiments may be performed to assess the development of disease symptoms and optimise time points and application methodologies. Field trials at different locations with larger plots are necessary to determine effects on yield and pathogen infection. For some diseases, artificial inoculation coupled with environmental manipulation (e.g. irrigation) may be necessary since natural infection is unpredictable (e.g. for *Fusarium* head blight; Rojas *et al.*, 2018).

The information acquired will help in developing protocols for improving the reliable use of endophytes. It is also biologically interesting to record the endophytic lifestyle under controlled/sterile conditions, to confirm they are not latent plant pathogens of the test or other crop, and confirm that they are not potential human pathogens (see Section 2.11).

2.10 Exploiting Endophytes as BCAs

Once biocontrol has been demonstrated, it is very important to study the mechanisms used by the organisms to reduce disease, i.e. mode of action. Interactions between plants and microbes are complex and there is currently no way of predicting whether a particular BCA will have a positive or negative influence on the ability of a specific pathogen to cause disease (Busby *et al.*, 2016b) and therefore we need to test for effect. The mechanisms contributing to biological control include antibiosis, parasitism, competition and induced resistance (Hardoim *et al.*, 2015; Ludwig-Müller, 2015; Jensen *et al.*, 2016b; Card *et al.*, 2016). Can individual mechanisms contribute in concert in a particular three-way interaction among plant, pathogen and antagonist? It is believed that components of microbial communities (e.g. rhizosphere and endosphere) also stimulate plant growth and thereby can act to control disease by strengthening the plant (Card *et al.*, 2016). It is generally believed that the most important mechanisms employed by endophytic BCAs are antibiosis and induced resistance. We address the mechanisms employed by endophytic BCAs in more detail elsewhere (Latz *et al.*, 2018).

An important potential advantage for an established endophyte is that it is better protected from the external environment than microorganisms applied to the phyllosphere or rhizosphere (Schulz and Boyle, 2005). Since endophytes are inside the plant, we can predict that some can provide effective and robust means for controlling disease, assuming that any effect as a BCA depends on it being an endophyte.

What comes out must be put back to be of use! It may not be easy to introduce the endophyte into a new plant and considerable developmental effort should be expected for potential products both to ensure reliable inoculation and stable effect under various environmental conditions. The challenge is greatest for field use (especially for foliar applications) and rather less for controlled conditions (greenhouse). Seed treatment is very attractive since it requires no further action by the grower. This may also be achieved by introducing the endophytic BCA to the flowers during pollination. Genetic variation in the host is also an important factor affecting endophyte colonisation and is an important aspect which needs to be addressed (Busby *et al.*, 2016b; Kroll *et al.*, 2017). Once introduced to the host, some endophytes can be transmitted through the seed, e.g. *Epichlöe* spp. (Rodriguez *et al.*, 2009; Card *et al.*, 2016). Some BCAs can be used as seed treatments, and can provide sustained control against root and even foliar infections. For example the protection afforded by *S. indica* in barley suggests maintenance of effects for weeks (Waller *et al.*, 2008). Aerial parts – leaves and flowers – are more sensitive to variation in environmental conditions, in particular, humidity and UV light can pose problems.

2.11 Concerns for the Use of Endophytes

There is clearly an ecological risk in moving endophytes between continents and it is not possible to predict a problem in advance. Thus, effects on natural microbial communities may possess a risk of causing trophic imbalance. Botanists and horticulturalists have moved many plant species and plant products, presumably with their accompanying microbiomes, for centuries apparently without ill effect. Occasionally, a catastrophe occurs, and plant pathology text books name the worst cases where pathogens have spread from an important introduced plant species to a native species with devastating effect. The mechanisms behind this are often obscure (Woolhouse *et al.*, 2005; Giraud *et al.*, 2010). An intriguing story has emerged with the relatively new disease ash dieback caused by the ascomycete fungal pathogen *Hymenoscyphus fraxineus* in European ash (*Fraxinus excelsior*). This disease emerged in the 1990s in the Baltic region and has subsequently spread west (McKinney *et al.*, 2014). The pathogen was first identified some 10 years after the disease was discovered and it was subsequently found that this is a natural endophyte or perhaps a weak pathogen in ash leaves of the eastern Asian species

Fraxinus mandchurensis. Perhaps native endophytes can be found which could be used to combat this disease (Kosawang *et al.*, 2018; Lahiri *et al.*, 2019, Chapter 15)?

That an endophyte can find a new host and become a pathogen after intercontinental transfer represents one risk, and it is clear that intercontinental transfer should in general be avoided or at least evaluated carefully. Are there other risks? Potential BCAs, including endophytes, should be tested thoroughly to ensure they are not pathogenic or otherwise harmful to the environment. Not easy! They may be present naturally in low amounts, but what happens if they are present in large quantities after application for agronomic purposes? One criterion could be to ensure that the BCA cannot persist for long in the environment in the absence of the host. However, there is a balance here. Should the product be difficult to use and inefficient, arguably it would be less attractive to the market than a more persistent (aggressive BCA). Whereas a non-persistent product may be attractive to the supplier, who could sell more, this option is less attractive to the grower.

A prerequisite for an endophytic BCA is that it should not be pathogenic towards humans (or allergenic or produce mycotoxins), even the immunocompromised, as it is very unlikely that these would be approved as products during the registration process. An early identification can be useful and with the ease of molecular identification, known human (and plant!) pathogens can be dropped from further study (Alabouvette *et al.*, 2012). A simple screen for pathogenic potential is to compare the growth in culture of the isolated endophytes at ambient temperature, where they would be used as BCAs or stimulants, with growth at 37°C or thereabouts. Ideally, growth should be prolific at the lower temperature as this would be useful for product formulation and production, but not at the latter temperature (Köhl *et al.*, 2011).

Finally, there is a myth that something natural is intrinsically safer than something that is artificial. Like other fungi, a BCA may produce mycotoxins or other harmful metabolites (Alabouvette *et al.*, 2012). The grass endophytes *Epichlöe* spp. are a case in point, and considerable effort has been put into developing mycotoxin-free *Epichlöe* BCA products (Card *et al.*, 2016). Part of the development of a potential BCA for use should therefore include checking for the production of potentially harmful metabolites.

The legislation concerning the use of BCAs in the EU follows the Registration of Biological Control Agents (REBECA) policy (Anon, 2009; Ehlers, 2011), and the Organisation for Economic Co-operation and Development (OECD) has provided guidelines to help harmonise legislation (Alabouvette *et al.*, 2012; OEDC, 2012). Currently there is an illogical discrepancy in the legal frameworks for using beneficial microorganisms in agriculture in some (mostly OECD) countries: an organism can be sold as a biofertiliser or biostimulant without addressing BCA activity, but all BCAs are subject to an extensive registration process and this is hampering the development of products (OEDC, 2012; Villaverde *et al.*, 2014; Anon, 2016).

2.12 Perspectives and Further Research

It is becoming increasingly clear that biological control will have to play a larger role in the plant protection programmes of the future. However, we still have much to learn. BCAs are often trickier to use than chemicals or disease resistance, but they can offer control solutions or contribute where chemicals and disease resistance are unavailable. We see an increasing role for endophytes in the BCA armoury. There are several areas which need to be focused on in order to achieve this; for example, the biology of the microorganisms concerned, including the potential for developing crops producing useful metabolites. Another important area concerns the development of reliable delivery systems for endophytes to the crop. Examples of delivery can include the ability to infect seed via the flower, seed coating or spraying the crop. Finally, we need a better understanding of the effects of host genotype and role of environment on specific disease risks. Most of all there are many exciting biological questions to be answered about the recruitment of endophytes, and concerning the nature of the interactions of endophytic microorganisms with each other, with other microorganisms in the plant (pathogens and the classic symbionts, e.g. mycorrhiza) and with the physiology of their hosts.

Consortia are combinations of organisms which can grow together giving an improved effect against a specific disease or perhaps several different beneficial effects. Small consortia can be designed to combine different microorganisms with different targets; for example, combining *Clonostachys rosea* with *Metarhizium brunneum* or *Beauveria bassiana*, which target fungal pathogens and insect pests, respectively (Kapongo *et al.*, 2008; Keyser *et al.*, 2016;). Consortia offer both advantages and disadvantages.

Advantages:

1. They may be designed to provide a combination of different modes of action to give broad-spectrum effect against several pathogens.
2. Organisms with different environmental optima may be combined to secure an effect under different environmental conditions.

Disadvantages:

1. Companies prefer single strains due to difficulties in approval.
2. They may not always give an enhanced effect (Xu *et al.*, 2011).

With the predicted 20% human population growth over the next 30 years, the effects of urbanisation and climate changes, global agricultural productivity needs a boost. Simultaneously, this boost must be implemented under higher environmental standards and global sustainability goals. Plant pathogens, pests and abiotic stress are agricultural challenges where microbiomes and specifically endophytes have shown potential for contribution to the alleviation of crop loss and stimulation

of crop yields. Our responsibility is to provide alternatives and to ensure that these achieve the potential by translating laboratory results into improved crop production globally.

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